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REVIEW

Engineering of chaperone systems and of the unfolded protein response

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Abstract Production of recombinant proteins in mammalian cells is a successful technology that delivers protein pharmaceuticals for therapies and for diagnosis of human disorders. Cost effective production of protein biopharmaceuticals requires extensive optimization through cell and fermentation process engineering at the upstream and chemical engineering of purification processes at the downstream side of the production process. The majority of protein pharmaceuticals are secreted proteins. Accumulating evidence suggests that the folding and processing of these proteins in the endoplasmic reticulum (ER) is a general rate- and yield limiting step for their production. We will summarize our knowledge of protein folding in the ER and of signal transduction pathways activated by accumulation of unfolded proteins in the ER, collectively called the unfolded protein response (UPR). On the basis of this knowledge we will evaluate engineering approaches to increase cell specific productivities through engineering of the ER-resident protein folding machinery and of the UPR.

Keywords Endoplasmic reticulum associated protein degradation · Heterologous protein production · Molecular chaperone · Protein folding · Unfolded protein response

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Abbreviations

4E-BP1	4E-Binding protein 1
ADP	Adenosine diphosphate
AKT	AKR/J Mice transforming retroviral oncogene
AP-1	Activation protein 1
ASK1	Apoptosis signal-regulating kinase 1
Asn	L-Asparagine
ATF4	Activating transcription factor 4
ATF5	Activating transcription factor 5
ATF6α	Activating transcription factor 6α
ATF6β	Activating transcription factor 6β
ATG5	Autophagy-related 5
Atg8p	Autophagy-related 8 protein
ATG12	Autophagy-related 12
ATG16	Autophagy-related 16
ATP	Adenosine triphosphate
BAK	BCL-2 Homologous antagonist/killer
BAP	BiP-Associated protein
BAX	BCL-2-Associated X protein
BBF2	Box B-binding factor 2
BCL-2	B Cell leukemia/lymphoma 2
BH1	BCL-2 Homology domain 1
BH2	BCL-2 Homology domain 2
BH3	BCL-2 Homology domain 3
BH4	BCL-2 Homology domain 4
BIM	BCL-2 Interacting mediator of cell death

BiP	Heavy chain binding protein
BMF	BCL-2 Modifying factor
bZIP	Basic leucine zipper
cAMP	Cyclic adenosine monophosphate
CD154	Cluster of differentiation 154
Cdc48p	Cell division cycle 48 protein
CDP	Cytidine diphosphate
CHO	Chinese hamster ovary
CHOP	CCAAT/Enhancer-binding protein (C/EBP) homologous protein
cIAP1	Cellular inhibitor of apoptosis 1
CNC	Cap and collar
CoA	Coenzyme A
COPII	Coat Protein II
CRE	cAMP Response element
CREB3	CRE-Binding protein 3
CREB4	CRE-Binding protein 4
CREB-H	CREB Homolog
Cue1p	Coupling of ubiquitin conjugation to ER degradation 1 protein
DD	Death domain
DED	Death effector domain
DnaJ	2′-Deoxyribonucleic acid chain elongation J
DR3	Death receptor 3
DR6	Death receptor 6
DTT	1,4-DL-Dithiothreitol
E_1	Ubiquitin-activating enzyme
E_2	Factor eluted by DTT (ubiquitin-conjugating enzyme)
E_3	Eluted with high salt or high pH (ubiquitin ligase)
ECH	Erythroid cell-derived protein with CNC homology
EDEM1	ER Degradation enhancer, mannosidase α-like 1
EDEM2	ER Degradation enhancer, mannosidase α-like 2
EDEM3	ER Degradation enhancer, mannosidase α-like 3
eIF2α	Eukaryotic translation initiation factor 2α
eIF2B	Eukaryotic translation initiation factor 2B
EIF2AK3	eIF2α Kinase 3
eIF-4E	Eukaryotic translation initiation factor 4E
ER	Endoplasmic reticulum
ERAD	ER Associated protein degradation
ERdj1	ER DnaJ Protein 1
ERdj2	ER DnaJ Protein 2
ERdj3	ER DnaJ Protein 3
ERdj4	ER DnaJ Protein 4
ERdj5	ER DnaJ Protein 5
ERGIC-53	ER Golgi intermediate compartment protein of 53 kDa
ERK1	Extracellular signal regulated kinase 1
ERK2	Extracellular signal regulated kinase 2
ERO1-Lα	Ero1p-like α
ERO1-Lβ	Ero1p-like β
Ero1p	ER Oxidation 1 protein
ERp57	ER Protein of 57 kDa
ERSE-I	ER Stress response element I
ERSE-II	ER Stress response element II
Erv2p	Essential for respiration and viability 2 protein
FAD	Flavine adenine dinucleotide
FADD	FAS-Associated protein with a novel DD
GADD34	Growth arrest and DNA damage gene 34
GADD45β	Growth arrest and DNA damage gene 45β
Glc	D-Glucose
α-Glc I	α-Glucosidase I
α-Glc II	α-Glucosidase II
GlcNAC	2-*N*-Acetyl-D-glucosamine
GRP78	Glucose-regulated protein of 78 kDa
GRP94	Glucose-regulated protein of 94 kDa
GRP170	Glucose-regulated protein of 170 kDa
GrpE	Growth after phage induction E
Hac1p	Homologous to ATF/CREB1 1 protein
HEDJ	Human ER-associated DnaJ
HERP	Hyperhomocysteinemia-induced ER stress-responsive protein
hIAP2	Human inhibitor of apoptosis 2
HMG	3-Hydroxy-3-methylglutarate
Hrd1p	HMG-CoA reductase degradation 1 protein
Hrd3p	HMG-CoA Reductase degradation 3 protein
HSP40	Heat shock protein of 40 kDa
HSP70	Heat shock protein of 70 kDa
HSP90	Heat shock protein of 90 kDa
IAP	Inhibitor of apoptosis
IgG	Immunoglobulin G

IκB	Inhibitor of NF-κB
IKK	IκB Kinase
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
IRE1α	Inositol requiring 1α
IRE1β	Inositol requiring 1β
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
JNK	JUN *N*-Terminal kinase
JUN	Ju-nana
KEAP1	Kelch-like ECH-associated protein 1
LC3	Microtubule-associated protein 1 light chain 3
LDLR	Low density lipoprotein receptor
Lhs1p	Luminal HSP70 1 protein
m^7G	7-Methylguanosine
Man	D-Mannose
MAP	Mitogen-activated protein
MAPKKK	MAP Kinase kinase kinase
MCP-1	Monocyte chemoattractant protein 1
MDG1	Microvascular differentiation gene 1
MEF	Mouse embryonic fibroblast
MKK7	Mitogen-activated protein kinase kinase 7
mRNA	Messenger RNA
mTNF	Membrane-bound TNF
MTJ1	Murine tumor cell DnaJ-like protein 1
mTOR	Mammalian TOR
NADPH	Reduced nicotine adenine dinucleotide phosphate
NF-κB	Nuclear factor κB
NOXA	NADPH Oxidase activator
NRF2	Nuclear factor erythroid 2-related factor 2
NS0	Non-secreting myeloma cell
OASIS	Old astrocyte specifically induced substance
ORP150	150 kDa Oxygen-regulated protein
p38	Protein of 38 kDa
$p58^{IPK}$	58 kDa Inhibitor of PKR
p65	65 kDa Protein
$p70^{S6K}$	70,000 M_r 40 S Ribosomal protein S6 kinase
p97	Protein of 97 kDa
PDI	Protein disulfide isomerase
PDK	Phosphoinositide-dependent kinase 2
PEK	Pancreatic eIF2α kinase
PERK	PKR-Like ER kinase
P_i	Inorganic phosphate (HPO_4^{2-})
PI	Phosphatidylinositol
PI3K	PI 3-Kinase
PKB	Protein kinase B
PKR	Double-stranded RNA-activated protein kinase
PP1	Protein phosphatase 1
PP_i	Pyrophosphate ($HP_2O_7^{3-}$)
PPI	*Cis–trans* peptidyl prolyl isomerase
pQC	Preemptive quality control
PUMA	p53 Upregulated modulator of apoptosis
Q6	Quiescin 6
QSCN6	Quiescin 6
QSCN6L1	Quiescin 6-like 1
QSOX1	Quiescin Q6 sulfhydryl oxidase 1
QSOX2	Quiescin Q6 sulfhydryl oxidase 2
RAS	Rat sarcoma
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNAi	RNA Interference
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
S1P	Site 1 protease
S2P	Site 2 protease
SEC61	Secretory 61
SEC63	Secretory 63
Ser	L-Serine
SERCA	Sarcoplasmic or endoplasmic reticulum Ca^{2+} ATPase
SH-2	Src Homology 2 domain
SHC	SH-2 Domain containing
SIL1	Suppressor of *IRE1*/*LHS1* synthetic lethality 1
SODD	Silencer of death domains
SOXN	Neuroblastoma-derived sulfhydryl oxidase
Src	Sarcoma formation
SRE	Sterol response element
sTNF	Soluble TNF
TACE	TNF-α Converting enzyme
Thr	L-Threonine
TIM	TRAF-Interacting motif
TNF-α	Tumor necrosis factor α
TNF-R	TNF-α receptor
TOR	Target of rapamycin
TRADD	TNF-R-Associated via DD

TRAF2	TNF-R Associated factor 2
TRAIL-R1	TNF-Related apoptosis-inducing ligand receptor 1
TRAIL-R2	TNF-Related apoptosis-inducing ligand receptor 2
TRB3	Tribbles homolog 3
tRNA	Transfer RNA
TSC1	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2
Ub	Ubiquitin
Ubc7p	Ubiquitin-conjugating enzyme 7 protein
UDP	Uridine diphosphate
UGGT	UDP-Glucose:glycoprotein glucosyltransferase
UPR	Unfolded protein response
VCP	Valosin-containing protein
WT	Wild type
XBP-1	X-Box-binding protein 1
Yos9p	Yeast osteosarcoma 9 homolog protein

Introduction

The world market for biopharmaceuticals is currently 48 billion US$ and has been growing annually by 19% over the last five years. Most biopharmaceuticals are therapeutic proteins, such as erythropoietin, blood coagulation factors, and monoclonal antibodies (Werner 2004). Mammalian cells are often the host of choice because of their ability to correctly fold, assemble, glycosylate, and secrete these proteins. Correct folding and a posttranslational modification pattern identical to the human protein are critical determinants of the activity, immunogenicity, and in vivo clearance rates of therapeutic proteins. Monoclonal antibodies are administered at high doses ranging from 25–40 mg every other week to 100 mg per day (Werner 2004). Protein production at multi-kilogram scales to sustain such therapies is required. The advent of these therapies has triggered speculations about capacity shortages and has highlighted that even modest increases in product yield will significantly decrease capital investment and the cost of goods (Werner 2004).

For all these reasons improvement is desirable at the upstream and downstream stages of the production process. Up to ~50% of the total production costs are associated with the upstream side, providing a strong rationale for upstream process engineering. Over the last decade product titers have increased from ~20 mg L^{-1} to 2 g L^{-1} (Werner 2004, Wurm 2004). These increases have been largely driven by increases in viable cell densities. Cell specific productivities nowadays usually reach 30–80 pg $cell^{-1}$ day^{-1} (Dinnis and James 2005). A plasma cell secretes several thousand IgM molecules s^{-1} (de StGroth and Scheidegger 1980; Randall et al. 1992), indicating that productivities of 200–400 pg^1 $cell^{-1}$ day^{-1} and 2.5–12 fold increases in cell specific productivities are possible, which will translate into significant savings in capital investment and the cost of goods. Considering the already high cell specific productivities of many (Dinnis and James 2005; Robinson and Memmert 1991; Wurm 2004), but not all (Ailor and Reff 2005), antibody expression systems, engineering of ER-resident chaperone systems and the UPR to increase cell specific productivities will be especially important for production of non-antibody proteins in animal cells, where cell specific productivities tend to be much lower (Hippenmeyer and Highkin 1993; Jeon et al. 2003; Lee et al. 2001; Park et al. 1999; Zang et al. 1995). For these reasons, we will summarize our understanding of ER-resident protein folding machineries and of the UPR, and discuss animal cell engineering approaches aimed at improving cell specific productivities by engineering of these protein folding machineries and of the UPR to alleviate a potential bottleneck in eukaryotic protein production factories, the folding and processing of the polypeptide chain in the endoplasmic reticulum (ER).

The rate- and yield limiting step for heterologous protein production

Several lines of evidence suggest that folding and posttranslational modification of newly synthesized proteins in the ER becomes rate- and yield-limiting in many eukaryotic expression systems as cell specific productivities approach the productivities of plasma cells. For example, the amount of secreted heterologous protein does not increase proportionally with gene copy number (Parekh et al. 1995), mRNA (Barnes et al. 2004; Fann et al. 1999; Pendse et al. 1992; Schröder et al. 1999), or even the intracellular

amount of the heterologous protein (Pendse et al. 1992; Schröder and Friedl 1997). Further, intracellular aggregation (Hasemann and Capra 1990; Hsu and Betenbaugh 1997; Hsu et al. 1996; Hsu et al. 1994; Schröder et al. 2002), association of heterologous proteins with the molecular chaperone BiP/GRP78 (Dorner et al. 1987; Hurtley et al. 1989; Jarvis and Summers 1989; Kaufman et al. 1988; Machamer et al. 1990; Suzuki et al. 1991), and dilation of the ER (Dorner et al. 1989; Gennaro et al. 1991) have been reported. BiP induction, a hallmark of ER stress and UPR activation, has been reported for many expression systems (Alete et al. 2005; Downham et al. 1996; Jones et al. 2005; Miura et al. 2001; Smales et al. 2004). Further, even in secretory cell types such as plasma cells activation of the UPR has been observed (van Anken et al. 2003; Zhang et al. 2005). These data demonstrate that the rate- and yield limiting step for production of recombinant secretory proteins in many eukaryotic expression systems is exit of the correctly folded polypeptide chain from the ER and highlight the critical roles of the UPR in professional secretory cells and in engineering of host cells with increased cell specific productivities.

The ER-resident protein folding machinery

In mammalian cells the ER functions as Ca^{2+} store, plays important roles in intracellular Ca^{2+} signaling, and is the site for the synthesis of phospholipids, sterols, secretory and transmembrane proteins (Bernales et al. 2006; Ron and Walter 2007; Schröder 2008a). The ER harbors a protein folding machinery that folds client proteins, quality controls the folding process, and activates the UPR to maintain the balance between the folding capacity of the ER and the folding demand imposed on this organelle (Strudwick and Schröder 2007). This folding demand arises from nascent polypeptide chains entering the ER in an unfolded conformation through the SEC61 translocation channel. Two classes of proteins, molecular chaperones, and protein foldases assist protein folding in the cell. Molecular chaperones interact with hydrophobic surfaces on unfolded proteins, thereby shielding them from engaging in non-productive interactions with other polypeptide chains. Chaperone foldases promote folding of client proteins through ATP consuming cycles in which they repeatedly bind to and dissociate from their substrates. Chaperone holdases bind to their substrates. Structural maturation of the substrate terminates the holdase-substrate interaction. The protein foldase families of protein disulfide isomerases (PDIs) and *cis–trans* peptidyl prolyl isomerases (PPIs) catalyze the formation and isomerization of disulfide bonds and the *cis–trans* isomerization of peptidyl prolyl bonds, respectively. Molecular chaperones and protein foldases participate in protein folding quality control, allowing only correctly folded proteins to exit the ER. Proteins recognized as unfolded are retained in the ER, and if judged to be folding incompetent removed from the folding cycles and targeted for proteasomal degradation in a process called ER associated protein degradation (ERAD). Autophagy also contributes to disposal of aggregated ER luminal proteins (Perlmutter 2006).

ER-resident molecular chaperone systems

Three general chaperone systems operate in the ER (Strudwick and Schröder 2007). The HSP70 chaperones BiP/GRP78 and Lhs1p/GRP170/ORP150 and their co-chaperones constitute the first, the HSP90 chaperone GRP94 the second, and the lectin chaperones calnexin, calreticulin, and calmegin the third chaperone system (Fig. 1). BiP assists completely unfolded polypeptide chains to fold, is involved in translocation of nascent polypeptide chains into the ER, gating of the SEC61 translocation channel, quality control of protein folding reactions in the ER, and activation of the UPR. GRP94 and the lectin chaperones preferentially interact with partially folded substrates. The viability of $grp94^{-/-}$ mouse embryonic fibroblasts (MEFs) (Biswas et al. 2007) suggests that GRP94 has a limited substrate spectrum.

BiP and Lhs1p—HSP70 chaperones of the ER

HSP70 chaperones consist of an *N*-terminal ATPase and a *C*-terminal substrate binding domain. In protein folding BiP cycles through rounds of ATP hydrolysis and ADP-ATP exchange reactions (Fig. 1). ATP hydrolysis and ADP-ATP exchange are stimulated by co-chaperones. DnaJ or HSP40 co-chaperones stimulate the ATPase activity of HSP70s. GrpE co-chaperones stimulate the ADP-ATP exchange reaction. At least six different DnaJ co-chaperones, ERdj1/MTJ1, ERdj2/SEC63, ERdj3/HEDJ, ERdj4/

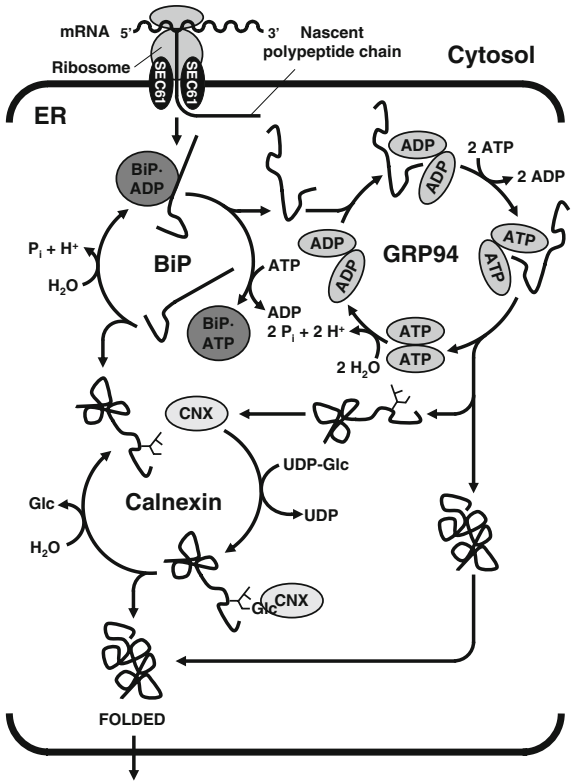

Fig. 1 Chaperone foldase systems in the mammalian ER. The relationship between the GRP94 and calnexin/calreticulin chaperone is not established. Abbreviations: Glc—D-glucose, P_i—inorganic phosphate (HPO_4^{2-})

MDG1, ERdj5, and $p58^{IPK}$, and one GrpE protein, BAP/SIL1, exist in the mammalian ER (Strudwick and Schröder 2007). HSP70 chaperones co-ordinate their functions. Lhs1p is a nucleotide exchange factor for BiP. BiP stimulates the ATPase activity of Lhs1p. In the ADP-bound form BiP has high affinity for unfolded substrates. Substrates bound to BiP are conformationally locked. ADP-ATP exchange releases the substrate from BiP, allowing the substrate to continue to fold. Substrate binding to BiP stimulates the ATPase activity of BiP, converting it to the ADP-bound form with high affinity for substrates. This ATP consumption cycle explains longstanding observations that protein folding consumes ATP (Dorner and Kaufman 1994).

GRP94—an HSP90 chaperone of the ER

HSP90 chaperones consist of an *N*-terminal ATPase domain, followed by a charged region, a dimerization domain and a *C*-terminal ER retention motif (Strudwick and Schröder 2007). The crystal structure of GRP94 is very similar to crystal structures of cytosolic HSP90s, suggesting a common mechanism of action (Dollins et al. 2007). In cytosolic and bacterial HSP90s ATP binding induces a closed conformation of the HSP90 dimer, in which the *N*-terminal domains of two protomers assemble into an active ATPase domain. This conformational change triggers substrate release. ATP hydrolysis and dissociation of ADP from HSP90s regenerate an 'open' conformation capable of interacting with client proteins (Fig. 1). GRP94, however, is insensitive toward the nature of the adenine nucleotide (Rosser et al. 2004) and adenine nucleotide binding does not trigger the change to the closed, ATP hydrolysis competent conformation, or substrate release (Rosser et al. 2004). Structural maturation of interacting substrates or regulatory interactions with yet to be identified co-chaperones may trigger substrate release and the ensuing conformational change in GRP94. What is then the role of the ATP hydrolysis cycle in GRP94 function? Whether GRP94 has ATPase activity has long been controversial (Dollins et al. 2007). Point mutations in the ATPase domain inactivate GRP94 (Randow and Seed 2001). GRP94 has a low ATPase activity that is comparable to the ATPase activity of yeast and human HSP90s (Dollins et al. 2007; Frey et al. 2007). The overall low ATPase activity of GRP94 may in part reflect the low probability of the chaperone sampling the hydrolytically productive closed conformation. Point mutations in the ATPase domain that diminish adenine nucleotide binding more severely compromise GRP94 function than point mutations predicted to render the ATPase domain inactive (Randow and Seed 2001). GRP94 is a labile protein that looses activity above 30 °C (Frey et al. 2007), which is well below the temperature optimum for mammalian cells, suggesting that adenine nucleotide binding stabilizes GRP94, and that ATP hydrolysis in the closed conformation provides the energy to return GRP94 into its open conformation to initiate a second round of substrate interaction.

The lectin chaperones calnexin, calreticulin, and calmegin

Many proteins entering the ER are posttranslationally modified by transfer of the oligosaccharide

$Glc_3Man_9GlcNAc_2$ (Glc—D-glucose, Man—D-mannose, GlcNAc—*N*-acetyl-2-D-glucosamine) to asparagine residues in the consensus sequence Asn-X-Ser/Thr (X = any amino acid except proline). Sequential action by two glucosidases, α-glucosidases I and II, removes the two terminal D-glucose residues (Fig. 2). The monoglucosylated species is recognized by the lectin chaperones calnexin, calreticulin, and the testis specific calnexin paralog calmegin/calnexin-t. These lectin chaperone foldases consist of an *N*-terminal globular lectin domain and a *C*-terminal extended hairpin loop, the P domain. Calnexin and calmegin are transmembrane proteins, calreticulin a soluble protein. Calnexin and calreticulin are chaperones for ~100 newly synthesized glycoproteins and have an overlapping, but not identical substrate spectrum (Williams 2006). The lectin domain recognizes monoglucosylated *N*-linked oligosaccharides. The tip of the P domain mediates association with the thiol oxidoreductase ERp57 (Williams 2006). The chaperone function is provided by both domains (Xu et al. 2004). Hydrolysis of the remaining third D-glucose residue from the oligosaccharide by α-glucosidase II terminates the substrate-chaperone interaction. If unfolded, the substrate is reglucosylated by UDP-glucose:glycoprotein glucosyl transferase (UGGT) triggering another round of interaction with calnexin or calreticulin (Fig. 2) (Dejgaard et al. 2004). This reglucosylation reaction consumes UDP-glucose and ultimately ATP. Proteins are extracted from the calnexin cycle by slow demannosylation reactions catalyzed by α-(1,2)-mannosidases I and II

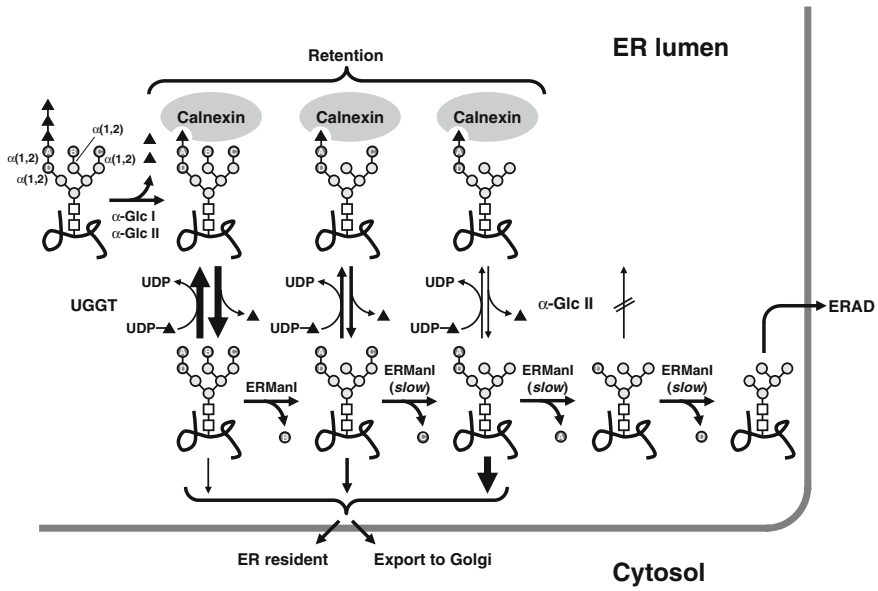

Fig. 2 Recognition of *N*-linked oligosaccharides as a marker for protein folding status (Lederkremer and Glickman 2005, Moremen and Molinari 2006). Oligosaccharyltransferase transfers the oligosaccharide $Glc_3Man_9GlcNAc_2$ (left, ▲—D-glucose, ○—D-mannose, □—2-*N*-acetyl-D-glucosamine) onto a newly synthesized polypeptide chain. α-glucosidases I and II (α-Glc I, α-Glc II) remove the two terminal D-glucose moieties. The monoglucosylated form is recognized by calnexin, calreticulin, or calmegin and retained in the ER. Upon removal of the terminal D-glucose moiety by α-glucosidase II, the protein is released from the lectin chaperone, but if still unfolded is reglucosylated by UDP-glucose:glycoprotein glucosyl transferase (UGGT). Removal of D-mannoses B and C converts the oligosaccharide into a poorer substrate for UGGT and α-glucosidase II and decreases the affinity of the oligosaccharide for calnexin and calreticulin and indirectly promotes export to the Golgi complex via recognition of oligosaccharides containing α(1,2)-linked D-mannose residues by export lectin receptors such as ERGIC-53. Trimming of D-mannose A removes the D-glucose acceptor from the oligosaccharide, efficiently blocks the reglucosylation reaction, inhibits the interaction with ERGIC-53, and triggers retrotranslocation and proteasomal degradation of slowly folding proteins. The demannosylation reactions are slower than the deglucosylation reactions. α(1,2)-ER mannosidase I preferentially removes D-mannose B, and more slowly the other D-mannose moieties. Golgi resident mannosidases exhibit specificity for D-mannoses A, C, and D and may be involved in trimming of the α(1,2)-linked D-mannose residues. Reprinted from Fig. 3 published in Schröder (2008a), copyright 2007, Birkhäuser Basel, with kind permission of Springer Science and Business

(Fig. 2). These demannosylation reactions generate oligosaccharide structures that serve as targeting signals for export from the ER or toward ERAD. Removal of mannoses B and C facilitates export to the Golgi complex via lectin receptors such as ERGIC-53 (Fig. 2). Removal of mannose A inhibits interaction with ER export receptors and targets the protein toward ERAD.

Oxidative protein folding in the ER

PDIs form a family of at least 17 proteins characterized by one or more CXXC amino acid motifs (Fig. 3a) (Ellgaard and Ruddock 2005). These proteins have thiol oxidase, disulfide isomerase, and chaperone activities. In *de novo* disulfide bond formation the oxidized form of a PDI is regenerated by disulfide bond shuffling between PDIs and FAD-dependent PDI-like proteins, yeast Ero1p, its mammalian orthologs ERO1-Lα and ERO1-Lβ, and Erv2p (Fig. 3b, c; Tu and Weissman 2002; Tu and Weissman 2004). Pdi1p is a poor substrate for Erv2p in vitro (Thorpe and Coppock 2007), suggesting that additional factors are involved in Pdi1p oxidation by Erv2p in vivo or that the physiological substrates of Erv2p are other redox proteins in the yeast ER. The final electron acceptors for Ero1p are molecular oxygen and to a lesser degree peroxide and superoxide (Tu and Weissman 2002). Two large mammalian transmembrane-anchored proteins, quiescin Q6/QSOX1/QSCN6 and QSOX2/QSCN6L1/SOXN, contain an Erv-like domain in addition to a thioredoxin domain (Fig. 3b; Thorpe and Coppock 2007). These sulfhydryl oxidases can directly transfer electrons from sulfhydryl groups to molecular oxygen via their thioredoxin and Erv-like domains. PDI is a poor substrate for QSOX in vitro. In vivo the bulk of PDI may be involved in disulfide bond isomerization requiring only a small amount of QSOX or ERO1-Lα/ERO-1Lβ for recycling PDI involved in disulfide bond formation (Thorpe and Coppock 2007). The ratio of oxidized to reduced PDI is 1:5 in vivo (Thorpe and Coppock 2007), supporting preferential engagement of PDI in disulfide bond isomerization. Alternatively, QSOX may directly introduce disulfide bonds into substrate proteins which are then reshuffled by PDI (Thorpe and Coppock 2007). Introduction of disulfide bonds into newly synthesized proteins has to intersect with conformational folding of the

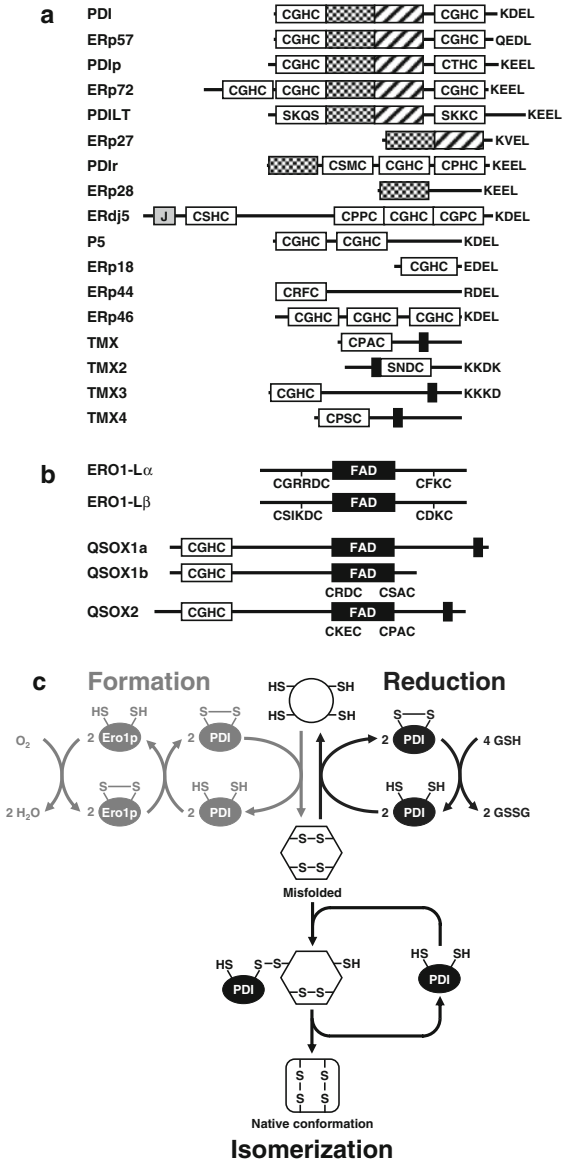

Fig. 3 Disulfide bond formation in the mammalian ER. (**a**) Human PDIs. Rectangles represent thioredoxin-like domains. Black rectangles represent transmembrane domains, rectangles labeled 'J' DnaJ domains, and labeled 'FAD' sequences involved in FAD binding. Catalytic site sequences and known ER retention signals are shown in the single letter amino acid code. (**b**) Human ERO and Erv-like proteins. QSOX1a and QSOX1b are products of alternative splicing. (**c**) Schematic of disulfide bind formation and isomerization reactions catalyzed by PDIs. Reprinted from (Schröder 2008b)

polypeptide chain. In vitro folding experiments favor a quasi-stochastic coupling mechanism in which formation of a conformationally stable set of disulfide bonds is followed by conformational folding of the

polypeptide chain (Welker et al. 2001). This mechanism of coupling of folding of the polypeptide chain to disulfide bond formation requires extensive collaboration between molecular chaperones and PDIs. Therefore, it is not surprising that many PDIs have chaperone activity or form stable complexes with molecular chaperones (Ellgaard and Ruddock 2005). A second important consequence of disulfide bond formation is generation of one molecule H_2O_2 for every disulfide bond formed. Disulfide bond formation accounts for ~25% of all reactive oxygen species (ROS) in an unstressed cell (Tu and Weissman 2004). During ER stress ROS accumulate in an *ERO1* dependent manner (Harding et al. 2003, Haynes et al. 2004). ROS adversely affect cell viability and possibly the quality of the protein product, trigger apoptosis, and function as signaling molecules in the UPR.

ERAD and autophagy

Correctly folded proteins are released from folding cycles and transported to the Golgi complex by packaging into COPII-coated vesicles at ER exit sites (Bonifacino and Glick 2004). For several secretory proteins cargo receptors have been identified. One example is ERGIC-53 which is required for ER to Golgi transport of blood coagulation factors V and VIII (Fig. 2; Nichols et al. 1998). Proteins that remain un- or misfolded after numerous folding attempts are extracted from the folding machinery to prevent its poisoning. These proteins are directed toward ERAD (Fig. 4a; Meusser et al. 2005). ERAD begins with recognition of a protein as unfolded. Chaperones and foldases, including BiP, calnexin/calreticulin, and PDI, and specialized lectins that recognize demannosylated *N*-linked oligosaccharides such as EDEM1-3 and the Yos9p·Hrd3p·Hrd1p ER membrane mannose lectin-ubiquitin ligase complex make this decision (Fig. 2) (Meusser et al. 2005). The roles of ER luminal HSP70 and HSP90 chaperones in selecting proteins for degradation is less well understood, but their cytosolic counterparts are known to interact with co-chaperones that target interacting clients to degradation (Alberti et al. 2003). A similar selection mechanism may operate in the ER. These degradation-promoting co-chaperones should be less abundant or display lower affinity interactions with their chaperone ATPases to allow for productive protein folding. The unfolded protein is transported to a retrotranslocation pore in the ER membrane. For many ERAD substrates this seems to be the SEC61 translocation channel (Fig. 4a), but alternative channels formed by Derlin proteins, or composite

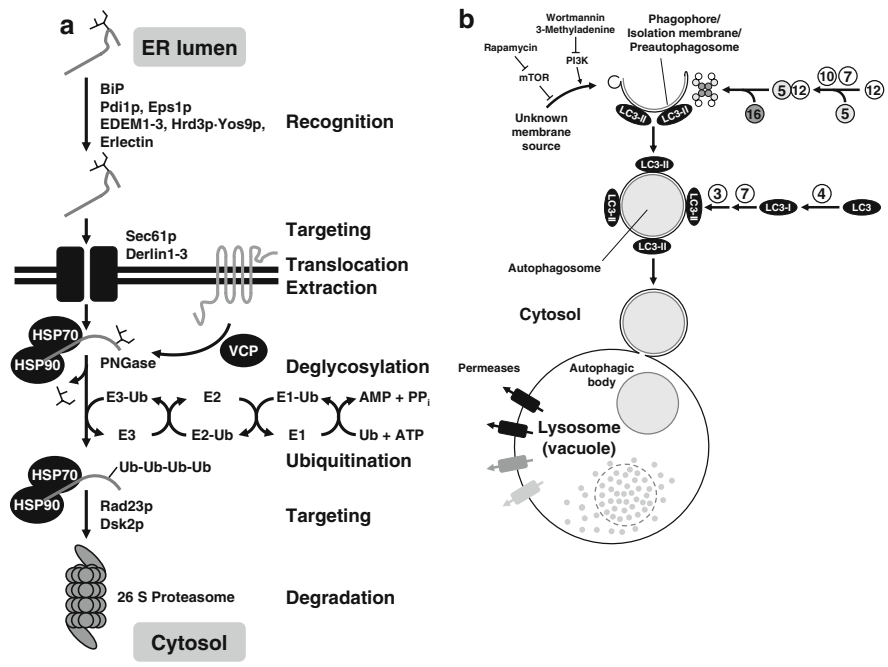

Fig. 4 ERAD (**a**) and autophagy (**b**). Formation of the phagophore is inhibited by TOR signaling and stimulated by a class III, wortmannin and 3-methyladenine sensitive PI3K. Abbreviations: PP_i—pyrophosphate, Ub—ubiquitin. Numbers in circles represent ATG proteins. Reprinted in modified form from Fig. 2 published in Schröder (2008a), copyright 2007, Birkhäuser Basel, with kind permission of Springer Science and Business

channels consisting of subunits of the SEC61 channel and Derlin proteins may also be involved. Valosin-containing protein (VCP)/p97/Cdc48p extracts proteins from the ER and the ER membrane (Meusser et al. 2005). In the cytosol the protein is deglycosylated and polyubiquitinated to direct it to the 26S proteasome. Cytosolic HSP70s and HSP90s interact with these doomed proteins to keep them in a soluble form to prevent their aggregation and precipitation. Ubiquitination is a three step process (Fig. 4a). An ubiquitin-activating enzyme (E_1) adenylates the ubiquitin *C*-terminus, covalently coupling ubiuquitin via a thioester to a cysteine residue in the activating enzyme. Ubiquitin-conjugating enzymes (E_2 s) and ubiquitin ligases (E_3 s) transfer activated Ub to the target protein via a thioester relay. Accessory proteins, such as HERP, recruit proteasomes to the ER membrane (Schröder 2008a, Strudwick and Schröder 2007).

A second degradative process recently implicated in clearance of aggregated ER luminal proteins is autophagy (Fig. 4b; Perlmutter 2006). In autophagy a membrane structure called the preautophagosome, phagophore or isolation membrane is formed from an unknown membrane source and engulfs bulk cytosol and whole organelles (Klionsky 2005; Tanida et al. 2004). Vesicle maturation involves two ubiquitination-like protein modification systems, which decorate the membranes of preautophagosomes with two ubiquitin-like proteins, Atg8p/LC3-I, and a tetrameric ATG12·ATG5·ATG16 complex. Lipidated Atg8p/LC3-I (called LC3-II) is also found on the membranes of mature autophagosomes. Mature autophagosomes fuse with lysosomes or vacuoles in which their content is degraded by digestive lysosomal or vacuolar enzymes.

The unfolded protein response

The UPR is a signaling network that adjusts the capacity of the ER-luminal protein folding machinery to the folding demand imposed on this machinery by coordinating the expression of many, if not all, genes encoding components of this machinery, but also induces immune, inflammatory, and apoptotic responses to ER stress [see (Bernales et al. 2006; Marciniak and Ron 2006; Ron and Walter 2007; Schröder 2006; Schröder 2008a, b; Schröder and Kaufman 2005a, b; Schröder and Kaufman 2006) for recent reviews]. In the following sections we summarize signal transduction mechanisms employed by the UPR and physiological response to ER stress activated by the UPR.

Signal transduction mechanisms in the UPR

The ER membrane of higher eukaryotes harbors at least three transmembrane proteins activated by ER stress, basic leucine zipper (bZIP) transcription factors synthesized as type II transmembrane proteins such as ATF6α and ATF6β, the protein kinase PERK/PEK/EIF2AK3, and the protein kinase-endoribonucleases IRE1α and IRE1β (Fig. 5). Additional type II transmembrane bZIP transcription factors resident in the ER membrane are CREB3, CREB4, CREB-H,

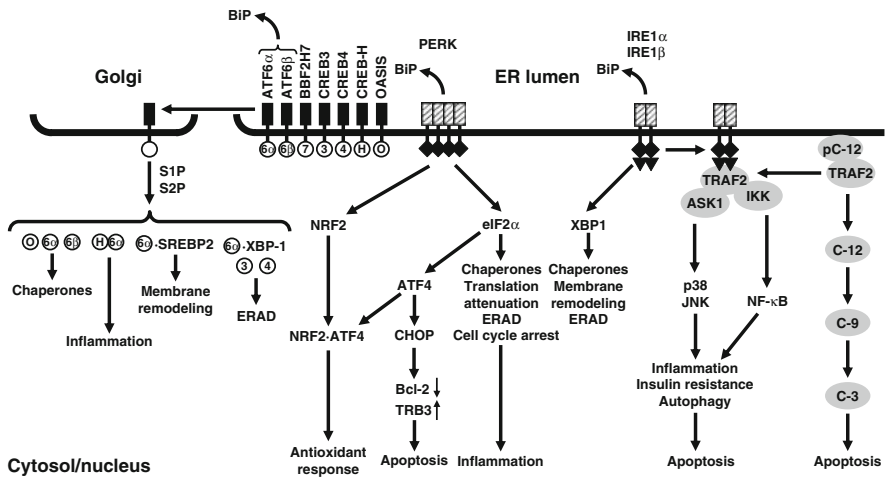

Fig. 5 Principal signal transduction pathways in the mammalian UPR. *Abbreviations*: C-3, caspase 3; C-9, caspase 9; C-12, caspase 12; pC-12, procaspase 12. Reprinted in modified form from Fig. 6 published in Schröder (2008a), copyright 2007, Birkhäuser Basel, with kind permission of Springer Science and Business

BBF2, and OASIS. BiP is associated with all stress sensors in unstressed cells and released from their ER luminal domains of these stress sensors upon exposure of cells to ER stress, which coincides with their activation.

ATF6 translocates to the Golgi upon release of BiP. In the Golgi sequential action by two proteases, site-specific proteases 1 and 2 (S1P and S2P), releases the cytosolic domain of ATF6 encompassing its bZIP and transcriptional activation domains (Fig. 5). This ATF6 fragment translocates to the nucleus to induce expression of chaperone genes. ATF6 activates target genes in concert with nuclear factor Y. S1P and S2P also activate the other type II transmembrane bZIP transcription factors. OASIS contributes to activation of the *BiP* gene, and ATF6 CREB-H heterodimers activate transcription of genes encoding acute phase proteins.

PERK oligomerizes and activates its protein kinase domain when BiP is released from its ER luminal stress sensing domain. Two substrates for the protein kinase activity of PERK are known, the bZIP transcription factor NRF2 and eukaryotic translation initiation factor 2α (eIF2α) (Figs. 5 and 6). In unstressed cells NRF2 is held in the cytosol in an inactive complex with the cytoskeletal anchor protein KEAP1. Phosphorylation of NRF2 by PERK disrupts this complex and triggers relocalization of NRF2 to the nucleus. A NRF2·ATF4 heterodimer activates an antioxidant response (Cullinan and Diehl 2006). Phosphorylation of eIF2α by PERK attenuates general cap-dependent translation by inhibiting loading of the translation initiation factor eIF2 with GTP, and formation of the 43 S preinitiation complex consisting of the 40S ribosomal subunit, GTP-bound eIF2, and methionyl methionine initiator tRNA. One consequence of translational arrest is clearance of short-lived proteins from the cell. Loss of D-type cyclins arrests the cell cycle in G_1. Loss of inhibitors of nuclear factor κB (NF-κB) (IκBs) activates NF-κB. Translational arrest in the UPR is transient. Even during translational arrest some mRNAs escape translational inhibition. Translation of mRNAs encoding short upstream open reading frames is stimulated when phosphorylation of eIF2α is increased (Fig. 6; Strudwick and Schröder 2007). An example for such an mRNA is *ATF4* mRNA which encodes a bZIP transcription factor. ATF4 activates an antioxidant response (Harding et al. 2003) and induces the inhibitor of mRNA 5′ cap-binding protein 4E-BP1 (Yamaguchi et al. 2008), which contributes to translational arrest during ER stress. The other two escape mechanisms are leaky scanning and internal initiation via internal ribosomal entry sites (Strudwick and Schröder 2007). Only a few mRNAs are known whose translation is stimulated in the UPR. These are the mRNAs encoding ATF4, ATF5 (Zhou et al. 2008), BBF2 (Kondo et al. 2007), and cellular inhibitor of apoptosis 1 (cIAP1)/human inhibitor of apoptosis 2 (hIAP2) (Warnakulasuriyarachchi et al. 2004; Yoshimura et al. 2008). Translational attenuation by

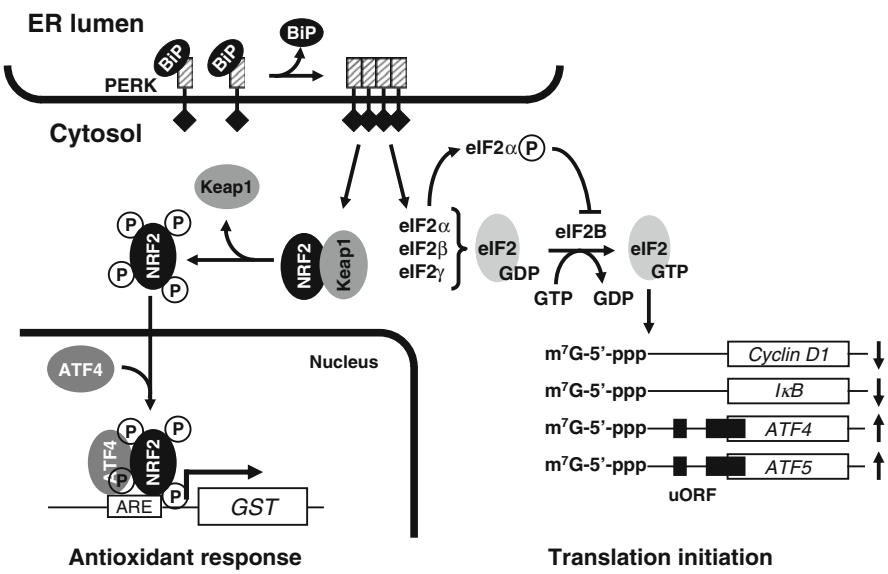

Fig. 6 Signal transduction by PERK. Abbreviation: m^7G—7-methylguanosine. Reprinted from *Current Molecular Medicine*, copyright 2006, with permission from Bentham Science Publishers

PERK is temporary. PERK signaling is attenuated by GADD34, a regulatory subunit of protein phosphatase 1 (PP1). GADD34 is activated late in the ER stress response and directs PP1 toward eIF2α to limit the time window of translational arrest. *gadd34*$^{-/-}$ cells fail to induce *ERO1α* and *WOLFRAMIN 1*, two genes whose protein products have well established functions in protein folding in the ER (Marciniak et al. 2004), and are protected from cell death triggered by ER stress (Zinszner et al. 1998). These observations suggest that recovery from translational arrest restores secretory capacity to cells at the price of increased susceptibility to ER stress and subsequent cell death.

Activation of IRE1 induces non-spliceosomal splicing of mRNAs encoding the bZIP transcription factors Hac1p in *Saccharomyces cerevisiae* and XBP-1 in metazoans (Fig. 5). IRE1 itself provides the endoribonuclease activity to cleave the exon-intron junctions in these mRNAs. The exons are ligated by an RNA ligase, tRNA ligase in yeast, and an unknown ligase activity in mammalian cells. Both transcription factors activate expression of genes encoding ER-resident molecular chaperones, protein foldases, and genes involved in ERAD, and phospholipid biosynthesis. In mammals, phosphorylated IRE1 assembles a pro-inflammatory and pro-apoptotic signaling complex. Phosphorylated IRE1 sequesters tumor necrosis factor receptor associated factor 2 (TRAF2) from pro-caspase 12 (Yoneda et al. 2001), resulting in clustering and activation of this caspase and activation of a caspase cascade (Fig. 5). TRAF2 recruits apoptosis signal-regulating kinase 1 (ASK1) and IκB kinase (IKK) to IRE1 (Hu et al. 2006; Nishitoh et al. 2002) to activate these protein kinases. ASK1 activates two mitogen-activated protein (MAP) kinase modules leading to activation of the MAP kinase p38 and jun *N*-terminal kinases (JNK) (Fig. 5). These kinases regulate immune, inflammatory, and apoptotic responses (Roux and Blenis 2004, Schröder 2008a). Association of the pro-apoptotic BCL-2 family proteins BAX and BAK with the cytosolic portion of IRE1 is required for efferent IRE1 signaling, i.e. *XBP-1* mRNA splicing and JNK1 activation (Hetz et al. 2006).

Physiological responses regulated by the UPR

Adaptive responses induced by the UPR increase the protein folding capacity of the ER or decrease the protein folding demand imposed on this organelle (Bernales et al. 2006, Ron and Walter 2007; Schröder 2008a). These responses include induction of molecular chaperones and foldases (Dorner et al. 1992) and upregulation of phospholipid synthesis to increase the size of the ER (Sriburi et al. 2004). To decrease the unfolded protein concentration in the ER, the UPR attenuates synthesis of secretory proteins by inhibiting general translation and transcription of genes encoding secretory proteins (Al-Sheikh et al. 2004; Pakula et al. 2003), upregulates ERAD (Friedlander et al. 2000; Yoshida et al. 2003) and autophagy (Ogata et al. 2006; Sakaki et al. 2008), selectively degrades mRNAs encoding secretory proteins (Hollien and Weissman 2006) and proteins that are in the process of translocating into the ER (Fisher et al. 1997; Gusarova et al. 2001). ROS accumulation in ER-stressed cells is countered by an antioxidant response mounted by the transcription factors NRF2 and NF-κB (Cullinan and Diehl 2006; Pham et al. 2004). If these actions fail to restore ER homeostasis the UPR induces apoptosis (Schröder 2008a; Xu et al. 2005). Activation of immune and inflammatory responses by the UPR may contribute to combating infections associated with ER stress, removal of dead cells from tissues and organs, and serve as an organismal alarm signal.

Induction of molecular chaperones and foldases

ATF6α stimulates transcription of genes encoding ER chaperones and foldases by binding to activating transcription factor (ATF)/cAMP response elements (CRE) and the ER stress response elements I and II (ERSE-I and ERSE-II) in the promoters of these genes (Lee et al. 2003; Wu et al. 2007; Yamamoto et al. 2007). ATF6α·ATF6β heterodimers inhibit transcription of the *BiP* gene (Thuerauf et al. 2007). The other arms of the UPR are also required for full induction of ER chaperone genes. BiP induction is decreased in cells carrying a S51A mutation in eIF2α (Scheuner et al. 2001). XBP-1 is required for induction of several DnaJ co-chaperones including p58^{IPK}, HEDJ, and MDG1 (Lee et al. 2003). Overlap of XBP-1 and ATF6α targets suggests that these two transcription factors fulfill partially redundant functions (Lee et al. 2003). AT6α·XBP-1 heterodimers stimulate transcription of genes involved in ERAD (Wu et al. 2007, Yamamoto et al. 2007). ATF6α

activation precedes activation of XBP-1 (Yoshida et al. 2003), resulting in a phase shift from a folding-only to a folding and degradation response. OASIS and XBP-1 contribute to induction of genes encoding ER chaperones and foldases through ERSE-I sites (Lee et al. 2003; Strudwick and Schröder 2007).

Stimulation of phospholipid synthesis

The ER expands in ER-stressed cells (Dorner et al. 1989; Wiest et al. 1990), but in contrast to professional secretory cells the stressed ER is often dilated and does no longer form characteristic cytosolic reticular networks. ATF6 and XBP-1 have been linked to regulation of ER size and lipid synthesis. Cell size, organelle content, total protein synthesis, mitochondrial mass and function, and ribosome number increased in mature B cells overexpressing spliced XBP-1. Knock-down of XBP-1 slightly diminished the ER (Shaffer et al. 2004). XBP-1 induces synthesis of phosphatidylcholine, and increases activity of the CDP-choline pathway leading to an increased surface area and volume of the rough ER in NIH-3T3 cells (Sriburi et al. 2004). A heterodimeric complex of ATF6 and sterol response element (SRE)-binding protein 2 recruits histone deacetylase 1 to the SRE in the low-density lipoprotein receptor (LDLR) promoter (Zeng et al. 2004) to repress cholesterol synthesis. Inhibition of cholesterol synthesis by ATF6 increases the fluidity of the ER membrane to preserve the function of SERCA Ca^{2+} pumps (Li et al. 2004) and to conserve carbon units for gluconeogenesis (Zeng et al. 2004).

ERAD and autophagy

The UPR induces proteins involved in different steps of ERAD. Spliced XBP-1 induces EDEM, Derlin-1 and Derlin-2, and HERP (Yoshida et al. 2003). ATF6α also induces HERP and Derlin-3 (Wu et al. 2007), and ATF4 contributes to induction of HERP. Thus, all three branches of the UPR contribute to stimulation of ERAD to enhance the clearance of misfolded proteins and decreasing the protein load in the ER during ER stress. The overlap of ERAD targets for ATF6α and XBP-1 can be explained by action of an ATF6α·XBP-1 heterodimer on the promoters of these genes (Yamamoto et al. 2007). In yeast, the UPR also induces the ubiquitin-conjugating enzyme Ubc7p and its membrane anchor Cue1p and the ubiquitin ligase Hrd1p involved in ERAD (Friedlander et al. 2000).

The UPR also stimulates autophagy (Ogata et al. 2006). In *ire1α*$^{-/-}$ cells or cells treated with JNK inhibitors formation of autophagosomes in response to ER stress was inhibited showing that the IRE1-JNK pathway is required for ER stress-induced autophagy (Ding et al. 2007; Ogata et al. 2006). Polyglutamine-induced conversion of LC3-I to LC3-II is inhibited in cells homozygous for the S51A-eIF2α allele or expressing dominant-negative PERK (Kouroku et al. 2007). Activation of protein kinase C θ, independent of any of the known ER stress sensors, is also required for stimulation of autophagy in ER-stressed cells (Sakaki et al. 2008). Genetic abrogation of autophagy sensitized cells to ER stress, suggesting that autophagy plays an important role in survival of ER stress (Ogata et al. 2006), possibly through its involvement in clearance of polyubiquitinated proteins (Ding et al. 2007).

Two additional degradative mechanisms targeting translocating polypeptide chains and their mRNAs contribute to decreasing the unfolded protein load of the stressed ER. In *Drosophila* Schneider S2 cells, IRE1 selectively degrades mRNAs associated with ER membrane-bound polyribosomes (Hollien and Weissman 2006). The IRE1 consensus cleavage site in these mRNAs is markedly different from the IRE1 cleavage site in *HAC1* or *XBP-1* mRNA, but bears some similarity to the cleavage site of IRE1β in 28 S rRNA (Iwawaki et al. 2001). These observations, together with different substrate specificities of the two human IRE1 paralogs (Imagawa et al. 2008) suggest that substrate specificity of IRE1 can be altered by covalent post-translational modification or by recruitment of another RNase to IRE1.

The second mechanism, termed pre-emptive quality control (pQC) (Kang et al. 2006), extracts polypeptide chains whose translocation is stalled from the translocation channel and targets them to the proteasome. Cytosolic HSP70s and HSP90s are required for co-translocational degradation of apolipoprotein B100 (Gusarova et al. 2001), suggesting that a ratcheting mechanism similar to the mechanism employed to translocate polypeptide chains into the ER extracts pQC substrates from the translocation channel. The ribosome and the translocon form a tight junction that blocks access of probes applied at

its cytosolic side to the translocating polypeptide chain. Less efficient signal peptides present on pQC substrates weaken this junction (Kang et al. 2006). Thus, a stochastic sampling mechanism may operate in which gated access of cytosolic chaperones to a polypeptide chain engaged with the translocon generates a signal for extraction and proteasomal destruction. Slowly translocating polypeptide chains or polypeptide chains stalled at the translocon interact longer with the translocon, which increases their chances to become a pQC substrate.

Attenuation of protein synthesis

At least four mechanisms attenuate secretory protein synthesis and decrease the folding demand imposed on the ER in the UPR. The most prominent and best characterized of these mechanisms is phosphorylation of eIF2α by activated PERK which inhibits cap-dependent translation. Induction of 4E-BP1 by ATF4 and subsequent inhibition of the 5′-cap binding protein eIF-4E by 4E-BP1 contributes to translational inhibition (Yamaguchi et al. 2008). Transcription of genes encoding secretory proteins is inhibited in lower eukaryotes such as yeasts (Kimata et al. 2006), filamentous fungi (Al-Sheikh et al. 2004; Pakula et al. 2003), and plants (Martínez and Chrispeels 2003). The forth mechanism is attenuation of metabolic activity, which also decreases ROS in ER stressed cells. JNK activation via the IRE1-TRAF2-ASK1 arm leads to serine phosphorylation of insulin receptor substrates (IRS) 1 and 2 (Özcan et al. 2004). IRS serine phosphorylation inhibits IRS phosphorylation at tyrosine residues by the insulin receptor [reviewed in (Draznin 2006; Saltiel and Kahn 2001; Shulman 1999)], promotes IRS-1 degradation (Shah et al. 2004), and inhibits insulin signaling, a condition referred to as insulin resistance. IRS tyrosine phosphorylation triggers binding of SH-2 domain containing proteins, including phosphatidylinositol (PI) 3-kinase (PI3K), to IRS proteins and SH-2 domain-containing (SHC) proteins and their relocalization to the plasma membrane. There PI3K phosphorylates PI, PI-4-phosphate and PI-4,5-bisphosphate, resulting in accumulation of PI-3,4-bisphosphate and PI-3,4,5-trisphosphate in the plasma membrane, and tethering of phosphoinositide-dependent kinases (PDKs) 1 and 2 and several isoforms of protein kinase B (PKB/AKT) to the plasma membrane (White 2002). Induction of TRB3 by CHOP (Ohoka et al. 2005) and inhibition of AKT by TRB3 (Du et al. 2003) contributes to inhibition of insulin signaling. Activated PKBs control many cellular events that stimulate growth, including D-glucose transport, protein, and glycogen synthesis. PKB and PDKs activate the protein kinase mTOR which stimulates translation by phosphorylating the protein kinase $p70^{S6K}$ and the translation initiation inhibitor 4E-BP1 to stimulate translation. SHC proteins activate mitogenic signaling via RAS, RAF, and the MAP kinases ERK1 and ERK2 to stimulate cell growth and division. Mitogenic signaling is largely intact in cells suffering from insulin resistance. Constitutive active mTOR signaling in MEFs defective in the mTOR inhibitory TSC1 TSC2 complex activates the UPR (Ozcan et al. 2008), possibly because of unregulated translation in these cells.

Antioxidant response

ROS accumulate in ER-stressed $perk^{-/-}$ cells (Harding et al. 2003) and in ER-stressed wild type (WT) yeast cells (Haynes et al. 2004). *ire1*Δ and *hac1*Δ strains are protected from ROS accumulation (Haynes et al. 2004). Knock-down of *ero-1* in *Caenorhabditis elegans* by RNA interference (RNAi) decreased ROS accumulation (Harding et al. 2003), suggesting that elevation of ERO1 levels by the UPR offsets the cellular redox balance. In yeast, mitochondria are a second source of ROS in ER stressed cells (Haynes et al. 2004). ROS activate redox-sensitive transcription factors such as NF-κB, heat shock transcription factor 1, and NRF2 (Veal et al. 2007). Oxidation of catalytic cysteines in JNK phosphatases activates JNKs (Veal et al. 2007) and may contribute to the development of insulin resistance in ER stressed cells. NF-κB protects cells from tumor necrosis factor (TNF)-stimulated ROS accumulation through induction of ferritin heavy chain (Pham et al. 2004). MEFs deleted for the p65 subunit of NF-κB accumulate ROS during ER stress (Mauro et al. 2006), suggesting that NF-κB has an antioxidant function in the UPR. NRF2 resides within the cytosol of unstressed cells in association with the cytoskeletal anchor protein KEAP1 (Cullinan and Diehl 2006). Phosphorylation of NRF2 by PERK disrupts the NRF2·KEAP1 complex. Oxidation of cysteine residues in KEAP1 by ROS may also

contribute to NRF2 activation in ER stressed cells. Upon release from KEAP1 NRF2 translocates to the nucleus to activate transcription of antioxidant genes, genes involved in protein folding and degradation and immune responses, and genes promoting cell growth and survival (Cullinan and Diehl 2006).

Apoptotic response to ER stress

All arms of the UPR stimulate apoptosis via activation of CHOP. *chop*$^{-/-}$ cells are resistant to ER stress-induced apoptosis (Zinszner et al. 1998). ATF6 and XBP-1 contribute to induction of CHOP. CHOP represses expression of antiapoptotic BCL-2 (McCullough et al. 2001), induces expression of proapoptotic TRB3 (Ohoka et al. 2005), and of GADD34 and ERO1-Lα (Marciniak et al. 2004). However, ATF4 is the most potent activator of CHOP in the UPR (Scheuner et al. 2001). Activation of JNK by IRE1 in the UPR requires the MAP kinase kinase kinase (MAPKKK) ASK1 and promotes apoptosis (Nishitoh et al. 2002, Yokouchi et al. 2008). JNK exerts its proapoptotic effect through phosphorylating and activating proapoptotic BH3-only proteins such as

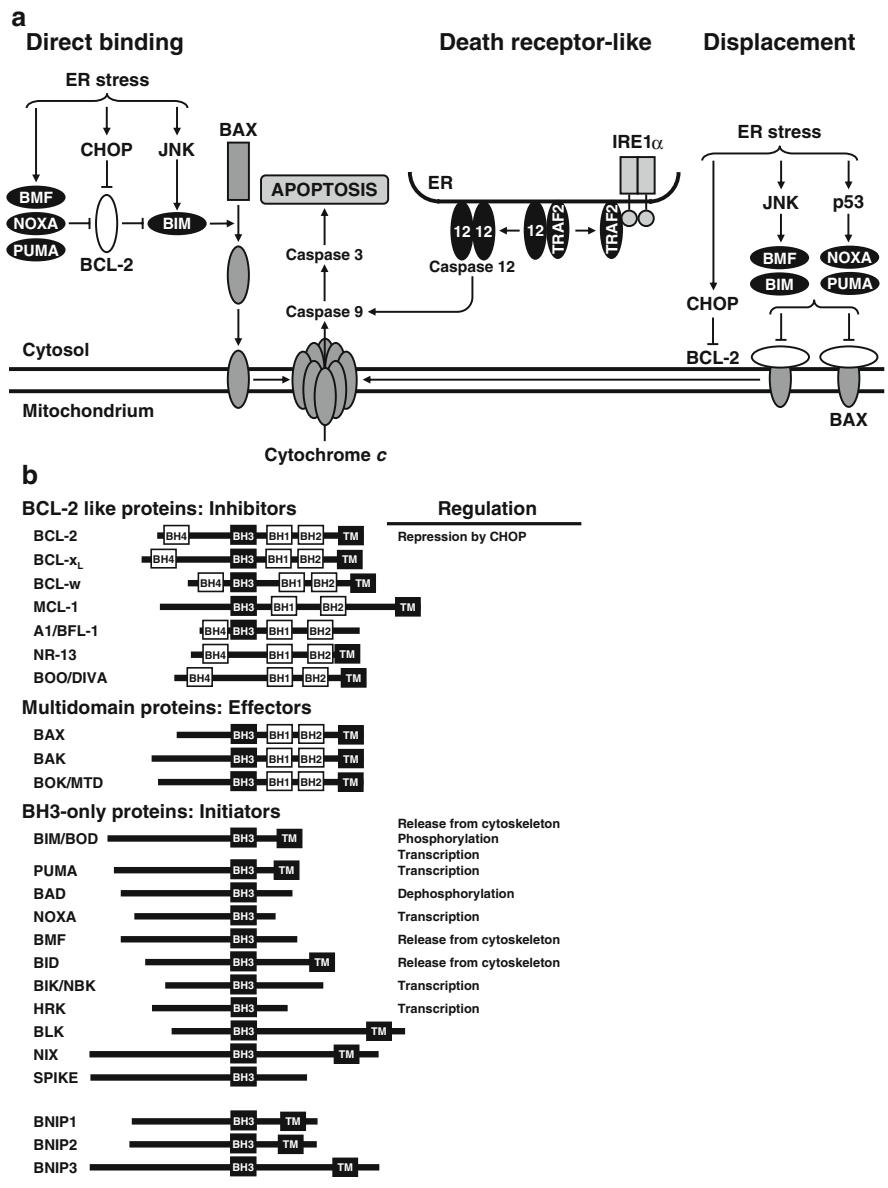

Fig. 7 Induction of apoptosis in the UPR. (**a**) Principal pathways according to the direct binding and the displacement models. In the direct binding model (left) proapoptotic BCL-2 proteins such as BIM associate with and activate BAK/BAX, leading to BAK/BAX oligomerization and insertion into the mitochondrial membrane, cytochrome *c* efflux, and activation of caspase 9. In the displacement model antiapoptotic BCL-2 proteins associate with and inactivate BAK/BAX. Proapoptotic BCL-2 family proteins, whose cytosolic concentration increases in response to intracellular damage, bind to and sequester antiapoptotic BCL-2 proteins from BAK/BAX, leading to BAK/BAX activation (Häcker and Weber 2007; Karst and Li 2007). Reprinted from Fig. 8 published in Schröder (2008a), copyright 2007, Birkhäuser Basel, with kind permission of Springer Science and Business. (**b**) Anti- and proapoptotic BCL-2 proteins and their regulation in the ER stress response. BH1, BH2, BH3, BH4, and transmembrane (TM) domains are represented by rectangles

BMF and BIM (Fig. 7a) (Schröder 2008a). Consistent with a proapoptotic role for JNK in the ER stress response is that selective activation of *XBP-1* splicing, but not of JNKs, increased survival and growth of cells exposed to drugs that induce ER stress (Lin et al. 2007). *traf2*$^{-/-}$ and *p65*$^{-/-}$ cells were more sensitive to ER stress than WT cells (Mauro et al. 2006). Activation of NF-κB by IRE1 through IKK (Hu et al. 2006) inhibits JNK through suppression of ROS (Pham et al. 2004) and induction of GADD45β which interferes with activation of the JNK kinase MKK7 (Papa et al. 2004). NF-κB induces cIAPs to suppress apoptosis (Wajant and Scheurich 2001). It is more difficult to understand why *traf2*$^{-/-}$ cells, in contrast to *ask1*$^{-/-}$ cells for example, are more sensitive to ER stress (Mauro et al. 2006). Both, TRAF2 and ASK1, are required for activation of p38, but TRAF2, through interaction with IRE1α mediates activation of IKK and NF-κB, which suppresses JNK-induced apoptosis. TNF-α, a type II transmembrane protein of the plasma membrane, is proteolytically released to the extracellular space and activates TNF-R signaling (Hu et al. 2006). Association of TRAF2 with TNF-R is required for the cytoprotective arm of TNF-R signaling through activation of IKK, NF-κB, and IAPs (Wajant and Scheurich 2001). Intact caspase activation by TNF-R in *traf2*$^{-/-}$ cells may be responsible for increased cell death of these cells in response to ER stress (Fig. 8).

IRE1 directly activates procaspase-12 by sequestering TRAF2 from procaspase-12 resulting in clustering and activation of caspase-12 at the ER membrane (Nakagawa et al. 2000; Yoneda et al. 2001). Caspase-12 initiates a caspase cascade leading to activation of the executioner caspase, caspase-3. *caspase-12*$^{-/-}$ MEFs are protected from ER stress induced cell death (Nakagawa et al. 2000). Human cells do not carry a functional *CASPASE-12* gene (Xu et al. 2005). In these cells caspase-4 may substitute for caspase-12. A role for caspase-4 in ER stress induced apoptosis is supported by localization of caspase-4 to the ER, its cleavage upon ER stress, and

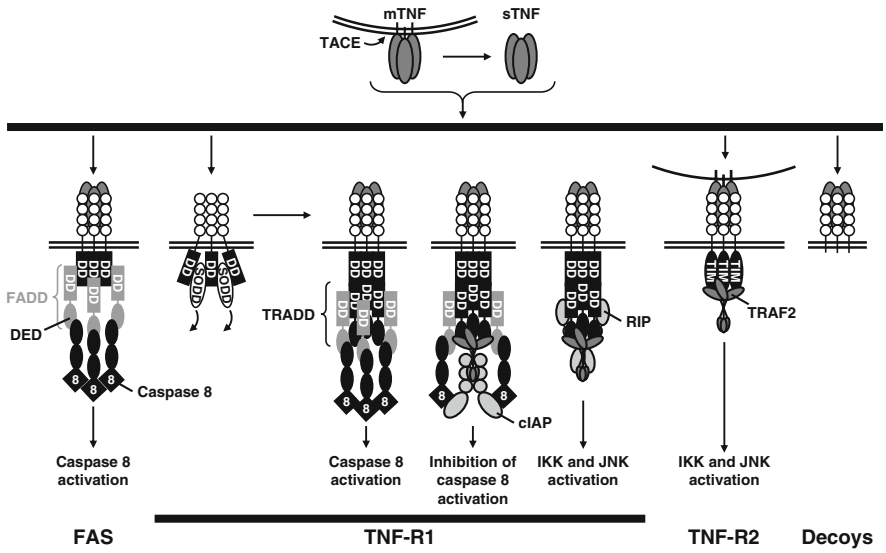

Fig. 8 Autocrine TNF signaling. Membrane-bound TNF (mTNF) is released from the plasma membrane by the protease TACE. Soluble (sTNF) or membrane-anchored TNF interacts with receptors of the TNF receptor (TNF-R) family. Interaction with death domain (DD)-containing receptors such as FAS, TRAIL-R1, and TRAIL-R2 recruits FADD and caspase-8 to the receptor and activates caspase-8. Other DD-containing receptors such as TNF-R1, DR3, and DR6 activate caspase-8 in a complex with TRADD. Conformational changes in the cytosolic domains of TNF-R1 induced by TNF binding release the inhibitory protein SODD from the cytosolic effector domains of the receptor. Binding of TRAF2 to the receptor TRADD complex either recruits cIAP to inhibit caspase-8 activation, or recruits IKK kinase and MAPKKKs such as ASK1 to activate NF-κB and the MAPK p38 and JNK. TRAF2-associated proteins such as RIP contribute to modulation of the outcome of TNF-R1 signaling. TNF-Rs with a cytosolic TRAF-interacting motif (TIM) domain directly associate with TRAF2 to activate IKK, p38, and JNK. TNF-R2 is preferentially activated by mTNF. Decoy receptors lack cytosolic effector domains and inhibit TNF signaling (Dempsey et al. 2003; Wajant and Scheurich 2001). Abbreviation: DED—Death effector domain

increased survival of ER stress by *caspase-4*$^{-/-}$ cells. Transcriptional induction of the proapoptotic BH3-only proteins BIM, PUMA, and NOXA, release of BIM and BMF from inactive cytoskeletal pools, and repression of transcription of antiapoptotic *BCL-2* by CHOP shifts the balance of pro- and antiapoptotic BCL-2 proteins toward antiapoptotic BCL-2 proteins and triggers apoptosis (Fig. 7b; Schröder 2008a).

Activation of immune and inflammatory responses

Immune and inflammatory signaling is activated by all three arms of the UPR. Proteolytic release of TNF-α from the plasma membrane to the extracellular space activates TNF-R signaling (Hu et al. 2006), leading to activation of NF-κB, and of the MAP kinases p38 and JNK (Fig. 8). NF-κB activates transcription of inflammatory genes such as TNF-α, TNF-β, interleukin (IL)-2, IL-6, and IL-8 (Baeuerle and Henkel 1994, Hu et al. 2006) and acute phase genes including serum amyloid A precursor, and complement factors B and C4 (Baeuerle and Henkel 1994). Activated JNK phosphorylates and potentiates the transcription factor AP-1, which induces expression of inflammatory cytokines including TNF-α, IL-6, IL-8, and MCP-1 (Gargalovic et al. 2006). A CREB-H ATF6 heterodimer induces inflammatory response genes such as serum amyloid P-component and C-reactive protein (Zhang et al. 2006).

Cell engineering of the early secretory pathway

Reports indicating that inefficient folding of heterologous proteins in the ER results in their intracellular aggregation (Hasemann and Capra 1990; Hsu and Betenbaugh 1997; Hsu et al. 1996; Hsu et al. 1994; Schröder et al. 2002) and association with BiP (Dorner et al. 1987; Hurtley et al. 1989; Jarvis and Summers 1989; Kaufman et al. 1988; Machamer et al. 1990; Suzuki et al. 1991), and, as a consequence, dilation of the ER (Dorner et al. 1989; Gennaro et al. 1991) has triggered interest in engineering of ER resident chaperone systems to boost the folding capacity of the ER of mammalian host cells through overexpression of chaperones and PDIs. These results have by and large yielded small if any increases in cell specific productivities. One possible explanation for these results is that overexpression of one chaperone may not be sufficient increase the folding capacity of the whole folding machinery in the ER and that coordinated elevation of expression of several chaperones and foldases may be necessary to obtain higher and more reproducible increases in cell specific productivities. Engineering of UPR signaling, for example through overexpression of one or a few of the transcription factors activated by the UPR provides an attractive avenue to achieve this goal and has attract considerable attention in the recent past. Results from the engineering of chaperone systems in insect and mammalian cells and of the studies reporting engineering of UPR signaling are reviewed in the following two chapters.

Engineering of the ER-resident protein folding machinery

Modeling of the BiP ATPase cycle predicts a modest increase in secreted IgG levels in BiP overexpressing cells (Whiteley et al. 1997). Such an increase has been observed in some studies (Hsu and Betenbaugh 1997; Whiteley et al. 1997). Other studies reported no effect or even a decrease in heterologous protein secretion (Dorner and Kaufman 1994; Dorner et al. 1988; Dorner et al. 1992; Hsu et al. 1994; Lambert and Merten 1997). Modeling also predicted 2-4 fold increases in secreted IgG levels when the ATPase cycle is slowed down, i.e. in the absence of ATP or the presence of non-hydrolyzable ATP analogs (Whiteley et al. 1997). A plausible explanation for this counter-intuitive result is that if ATP-binding and hydrolysis are slowed down, a larger proportion of BiP molecules will exist in the ADP-bound state that has higher affinity for unfolded substrates, thus preventing their non-productive aggregation. Slowing down of the BiP ATPase cycle is a likely consequence of BiP overexpression, as its co-chaperones or ATP itself may become limiting. The affinity of HSP70 chaperones for ADP is $\sim$6 times higher than for ATP. Thus, BiP exchanges ADP for ATP very slowly without catalysis. So why then is this predicted increase in recombinant protein secretion not consistently seen in BiP overexpressing cells? HSP70 chaperones, including BiP, do not work in isolation, but in networks with other HSP70 chaperones (Morano 2007). BiP and Lhs1p coordinate their folding efforts (Steel et al. 2004). Stalled ATPase cycles may be a degradation signal. Elevated BiP levels may increase

the prevalence of interactions with degradation-promoting co-chaperones or protein complexes. Increased folding capacity of the BiP ATPase cycle may stall the GRP94 or calnexin-calreticulin chaperone machineries. BiP overexpression attenuates the UPR (Dorner et al. 1992; Kohno et al. 1993), suggesting that BiP levels in WT and BiP overexpressing cells expressing a heterologous protein may not be different. Selective overexpression of BiP in cells experiencing ER stress may blunt induction of other chaperones, thereby causing an imbalance in ER luminal chaperone activities, a situation which may favor degradation. BiP overexpression induces ER-derived BiP-containing vesicles in the cytosol (van der Heide et al. 2002) or nucleus (Morris et al. 1997). These vesicles may be similar to 'BiP bodies' observed in yeast in which ER-to-Golgi transport is blocked (Nishikawa et al. 1994) and function in normal cells as cargo selection sites for ER-to-Golgi traffic (Nishikawa et al. 1994) or sites that target proteins for degradation (van der Heide et al. 2002).

Increasing expression levels of calnexin/calreticulin improved secretion of heterologous proteins in insect cells and Chinese hamster ovary (CHO) cells (Chung et al. 2004; Kato et al. 2005). These results are difficult to reconcile with overexpressed calnexin/calreticulin functioning in the calnexin cycle, because expression of all cycle constituents should be maintained at a ratio optimal for maximum cycle capacity. Therefore, increased levels of one cycle constituent should not improve cycle performance. Attractive alternative explanations are that the extra amount of calnexin or calreticulin functions as a chaperone holdase which buffers against an increased unfolded protein load, or that these extra amounts form inactive complexes with lectins such as EDEM1-3 which normally target unfolded proteins to ERAD.

Engineering of PDI expression levels gave a similar picture as described for BiP. Secretion of some heterologous proteins was increased (Kato et al. 2005; Lambert and Merten 1997), but secretion of an equal number of heterologous proteins was not affected (Kitchin and Flickinger 1995; Mohan et al. 2007). PDI may enhance secretion via increased rates of disulfide bond formation and isomerization, or because of its chaperone function. Overexpression of catalytically inactive PDI improved secretion of some heterologous proteins (Hayano et al. 1995), and likewise, overexpression of PDI also improved secretion of heterologous proteins containing no disulfide bonds (Smith et al. 2004), suggesting that PDI overexpression exploits its chaperone function and not its oxidoreductase or isomerase functions. This interpretation is more consistent with the concept that enzymes function at substoichiometric levels compared to their substrates and that efficient chaperone function requires at least equimolar amounts of chaperone and substrate.

Engineering of the UPR

Induction of BiP in several expression systems (Alete et al. 2005; Dorner et al. 1989; Downham et al. 1996; Jones et al. 2005; Miura et al. 2001; Smales et al. 2004; Watowich et al. 1991), of NF-κB in human IL-6 expressing CHO cells (Teruya et al. 2005), and of genes involved in the ER stress response including *BiP*, *GRP94*, *calnexin*, and *HERP* in human bone morphogenic protein 2 expressing CHO DUKX cells (Doolan et al. 2008) demonstrates that the UPR is activated in many heterologous expression systems. Cooperativity of molecular chaperones and foldases may necessitate increasing expression of several chaperones simultaneously to reproducibly increase productivities. This goal may be reached through constitutive activation of the UPR. Therefore, strong rationales exist to engineer the UPR to boost cell specific productivities. UPR and chaperone engineering may be of greater importance for slowly folding or unstable proteins as these proteins tend to induce the UPR more efficiently (Gething et al. 1986; Watowich et al. 1991). In mammalian cells, engineering of UPR signaling is complicated by existence of several signal transduction pathways (Fig. 5), extensive crosstalk between these pathways, and simultaneous activation of responses beneficial (induction of ER-resident chaperones and expansion of the ER) and detrimental (ERAD, attenuation of protein synthesis, apoptosis, and inflammation) to recombinant protein production. Activation of inflammatory signaling (Fig. 8) as a consequence of UPR activation in ER-stressed production cells or cells engineered for constitutive UPR activity is a concern with regard to product quality, and requires further investigation.

Boosting the beneficial aspects of the UPR, i.e. through overexpression of transcription factors such

as ATF6 or XBP-1 has received some attention in the recent past. Overexpression of XBP-1 in mammalian cells increased secretion of interferon γ, erythropoietin, a human monoclonal antibody, secreted alkaline phosphatase, and human vascular endothelial growth factor 121 (Ku et al. 2008; Tigges and Fussenegger 2006). These results may reflect dissection of UPR signaling beneficial for protein secretion from detrimental activities, such as translational attenuation by PERK and induction of apoptosis via PERK and JNK activation. However, these results are not generally applicable. Expression of an humanized anti-CD154 antibody in CHO DG44 cells was only modestly enhanced by individual or combined overexpression of the cytosolic ATF6 fragment, XBP-1, or a fragment of eIF2α carrying the S51A point mutation (Ailor and Reff 2005). Expression of human antithrombin III or granulocyte-macrophage colony-stimulating factor in CHO DXB11 cells was not enhanced by spliced XBP-1 (Ohya et al. 2007). XBP-1 was spliced in antithrombin III expressing CHO DXB11 cells even before introduction of spliced *XBP-1* cDNA (Ohya et al. 2007). This result illustrates that potential improvements achievable by engineering of the UPR may be masked by UPR activation in production cell lines. Induction of ERO1-Lα and ERO1-Lβ by XBP-1 elevates ROS (Harding et al. 2003; Haynes et al. 2004; Yokouchi et al. 2008). ROS may damage the heterologous protein product. NF-κB, but not BiP, activation in CHO cells expressing human IL-6 suggests that these cells suffer from oxidative stress (Teruya et al. 2005).

Equally attractive are attempts to abrogate responses detrimental for protein production activated by the UPR. Overexpression of S51A-eIF2α in CHO cells blocks translational attenuation mediated by eIF2α phosphorylation and increased expression of firefly luciferase $\sim$3-fold (Underhill et al. 2003). Guanine nucleotide exchange is catalyzed by eIF2B, which is $\sim$10 times less abundant in cells than eIF2α (Kaufman 2004). A potential limitation of this approach is that only small increases in eIF2α phosphorylation trigger complete translational arrest. Overexpression of ATF4 in antithrombin III-expressing CHO DXB11 cells decreased phosphorylation of eIF2α and improved secretion of antithrombin III (Ohya et al. 2007). ATF4 expression induced CHOP $\sim$2 fold, but did not decrease cell viability. GADD34 expression, an ATF4 target that directs PP1 toward eIF2α, was not significantly increased (Ohya et al. 2007). Manipulation of CHOP expression in NS0 cells did not alter survival of ER stress (Cudna and Dickson 2006), again showing that CHOP is not sufficient to trigger apoptosis in production cells.

Conclusions

In several mammalian expression systems, folding and maturation of the recombinant protein in the ER is the rate- and yield-limiting step for its production and activation of the UPR has been observed. Thus, clear rationales exist to engineer the UPR. Currently, only limited information is available on the effect of UPR engineering on cell specific productivities. However, this data already provides a mixed picture. Some studies have reported improvements, other studies reported no improvements or even decreases in cell specific productivities, a situation which is reminiscent to engineering of chaperone systems. Improvements obtained through engineering of the UPR and of chaperone systems may be masked by endogenous activation of the UPR in expression cell lines (Ohya et al. 2007). Another important aspect of UPR engineering will be to selectively activate those responses beneficial to protein production, while responses detrimental to protein production are not elevated or even abolished. Future engineering of the UPR will hopefully include these considerations, and lead to a more consistent outcome of UPR and chaperone system engineering, an achievement critical for routine chaperone and UPR engineering in commercial settings.

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